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Differentiation of Human Phospholipase A_2 Isoenzymes in Serum and Other Body Fluids with Use of Monoclonal Antibodies

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Summary: Elevated phospholipase A_2 activities in serum were measured in patients suffering from acute pancreatitis or various inflammatory diseases. The photometric phospholipase A assay of *Hoffmann & Neumann* (Klin. Wochenschr. *67* (1989) 106–109) was combined with immunoabsorption by different monoclonal antibodies directed against pancreatic phospholipase A_2. Pancreatic phospholipase A_2 was purified from human duodenal juice. Monoclonal antibodies were prepared by fusion of spleen cells from immunized mice with P3X63-Ag8-653 myeloma cells. Samples with phospholipase A_2 activity were incubated in monoclonal antibody-coated microtitre plates. Phospholipase A_2 activities were determined in the monoclonal antibody-treated samples as well as in control samples. The method allows the determination of the fraction of human phospholipase A_2 isoenzymes in various biological materials. For pancreatic phospholipase A_2 the specific binding capacity was about 60–80%, the unspecific binding was 5–30%. Practically no cross-reactivity was seen with partially purified serum phospholipase A_2, with recombinant platelet phospholipase A_2, or with the sera of patients with non-pancreatic diseases. In conclusion, the present study confirmed the presence of pancreatic phospholipase A_2 in human duodenal juice and in the ascites of necrotizing pancreatitis. However, pancreatic isoenzyme was absent in non-pancreatic inflammatory diseases. Therefore, elevated phospholipase activities in non-pancreatic inflammatory diseases cannot be attributed to the pancreas.

Introduction

Serum phospholipase A_2 (EC 3.1.1.4) has long been assumed to originate exclusively from the pancreas (for review see l. c. (1)). More recently, this assumption has been challenged by our group (1–4) and other authors (5–7).

In 1988, *Eskola* et al. (6) demonstrated the existence of two different forms of phospholipase A_2 in human serum using polyclonal antibodies against the pancreatic isoenzyme. Their study design, however, did not unequivocally prove that one of these "isoenzymes" was pancreatic phospholipase A_2; since phospholipases A_2 possess a highly conserved protein structure (8, 9), polyclonal antibodies against the pancreatic enzyme might cross-react with structurally related isoenzymes. Immuno-reactive proteins have been found in serum of patients with non-pancreatic malignant tumours (10) as well as in rat spleen (11). On the other hand, most phospholipases A_2 have a marked tendency to bind to proteins and surfaces in an unspecific manner (3, 12, 13), so that antibody-independent binding artifacts may occur.

The present article describes a practicable combination of activity measurements based on a photometric assay (2) and immunoabsorption by different monoclonal antibodies. The photometric assay has been

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shown to measure pancreatic and non-pancreatic phospholipase A_2 activity (4, 14) and to be equivalent to radiometric methods (14). The technique allows the determination of the fraction of human phospholipase A_2 isoenzymes, and is suitable for application to various biological materials.

Materials and Methods

Recombinant human platelet phospholipase A_2 (15) was a kind gift of Biogen Inc., Cambridge MA, USA. The enzyme solution (0.1 g/l) was diluted 50- to 100-fold with 10 mmol/l TRIS/HCl pH 8 containing 20 g/l bovine serum albumin.

Human pancreatic phospholipase A_2 was purified from duodenal juice an described previously (16) by a combination of precipitation steps, hydrophobic and cation exchange chromatography.

Human serum phospholipase A_2 was prepared from 0.7 l pooled serum of unselected patients of our hospital, using only the final cation exchange step of the latter purification procedure with slight modifications: the native serum was diluted 1 : 2 with 20 mmol/l TRIS/HCl pH 8.4 and the chromatography was performed in the same buffer. Active fractions eluted with 700 mmol/l KCl were pooled, dialysed against KCl-free buffer and lyophilized to dryness after addition of 50 g/l mannitol.

For the antibody binding experiments, native sera of individual patients were pooled separately, stored at 4 °C, and diluted with 20 mmol/l TRIS/HCl pH 7.5 containing 20 g/l bovine serum albumin to give a final activity of 50 to 70 U/l. The underlying diagnoses were necrotizing pancreatitis (patient A, female, 84 years), severe burns (patient B, male, 42 years), and septicaemia (patient C, male, 42 years). Further body fluids tested for antibody binding were native duodenal juice obtained during pancreas function tests as well as abdominal and pleural exudations of a patient D (male, 55 years) with a necrotizing bout of chronic pancreatitis.

Monoclonal antibodies directed against pancreatic phospholipase A_2 were produced, using purified phospholipase A_2 from human duodenal juice (see above) as an antigen. Balb/c mice, 12 weeks old, were immunized intraperitoneally with 50 µg antigen in complete *Freund*'s adjuvant. Two booster injections were given at monthly intervals under the same conditions. Three days before fusion, an intravenous injection of 50 µg antigen was given in saline without *Freund*'s adjuvant.

Fusion of spleen cells from immunized mice was performed with P3X63-Ag8-653 myeloma cells, and fused cells were cultivated in RPMI 1640 medium supplemented with fetal calf serum, volume fraction 0.1, 2 mmol/l *L*-glutamine and 1 mmol/l sodium pyruvate, containing 0.1 mmol/l hypoxanthine and 0.01 mmol/l azaserine as a selective medium.

Hybridoma supernatants were screened for binding to phospholipase A_2 adsorbed on microtitre plates (Nunc). Anti-phospholipase-antibodies were detected by sheep anti-mouse-Fcγ-antiserum labelled with horseradish peroxidase. ABTS substrate was added and the absorbance at 405/455 nm recorded in an ELISA-reader from Dynatech.

The antibodies were purified either by anion exchange (DE), cation exchange (Q), or size exclusion chromatography (TSK), biotinylated and provided as a lyophilized powder.

The following five lots out of eight different antibodies obtained, showed the highest relative affinity to pancreatic phospholipase A_2:

A: 1.185.175-IgG(DE)-Bi(X-Osu)
B: 2.106.154-IgG(Q)-Bi(X-Osu)
C: 2.50.75-IgG(TSK)-Bi(X-Osu)
D: 2.6.24-IgG(TSK)-Bi(X-Osu)
E: 2.223.288-IgG(Q)-Bi(X-Osu)

For our experiments, antibody solutions (10 mg/l) were prepared with buffer A (50 mmol/l potassium phosphate buffer pH 7.5 containing 150 mmol/l NaCl).

Streptavidin-coated microtitre plates (TRSA-SA) as well as the substrate kit for phospholipase A determination (cat. No. 1056239) were from Boehringer Mannheim, Germany. Other reagents were: bovine serum albumin from Serva, Heidelberg, Germany, poly-*L*-lysine hydrobromide from Sigma, St. Louis, USA, and the Coomassie protein test kit from Biorad, Anaheim, USA. All other reagents were of the highest commercial quality.

Immunoabsorption assay for pancreatic phospholipase A_2

The photometric activity assay was performed as described (17) with two exceptions: the fatty acid test kit from Wako Chemicals, Neuss, Germany (cat. No. 273-75409) was used as in l. c. (2) and pancreatic phospholipase A_2 was determined at pH 6.0 instead of 8.0 (16).

For preparation of antibody-coated microtitre plates, 0.2 ml antibody solution (10 mg/l) were pipetted into each well and discarded after incubation for 30 min at room temperature. In order to block non-specific binding sites of the plates a second incubation was performed with 0.2 ml of poly-*L*-lysine (0.1 g/l). The fluid was discarded after 30 min and the wells were rinsed three times with buffer A. Control plates were prepared using buffer A instead of antibody solution.

A phospholipase-containing sample (0.1 ml, activity 50–70 U/l) was incubated for 30 min at room temperature. Enzyme activities were determined at 37 °C in the untreated sample as well as in the antibody and the control incubation: 0.04 ml sample were added to 0.2 ml prewarmed substrate solution (37 °C). After exactly 5 and 20 min, 0.04 ml of the incubation mixture was transferred to 0.02 ml ice-cold stop solution. For fatty acid determination, 0.4 ml prewarmed Wako-reagent A were added to this stop mixture and 10 min thereafter 0.8 ml reagent B. The absorbance at 546 nm (1 cm path length) was read after another 10 min and the activity was calculated using a molar absorbance of 1540 m^2/mol (2):

$$\text{phospholipase } A_2 \text{ (U/l)} = (A_{20\,min} - A_{5\,min}) \times 818$$

Taking the activity of the untreated sample (u) as 1.0, the relative activities of the antibody-treated samples (a) and the control samples (c) were calculated. Total, specific, and unspecific binding (TB, SB, UB) were derived from these percent values by the following formulae:

$$TB = 1.0 - a$$
$$UB = 1.0 - c$$
$$SB = TB - UB = c - a$$

Results

In a first series of experiments, we tested the antigen binding capacity of the microtitre plates with purified pancreatic phospholipase A_2. Figure 1 shows the results for those two antibodies which were best suited for the differentiation between pancreatic and non-pancreatic phospholipase A_2 in biological fluids (see also tab. 1). Using the antigen solution under the standard conditions described in the methods section,

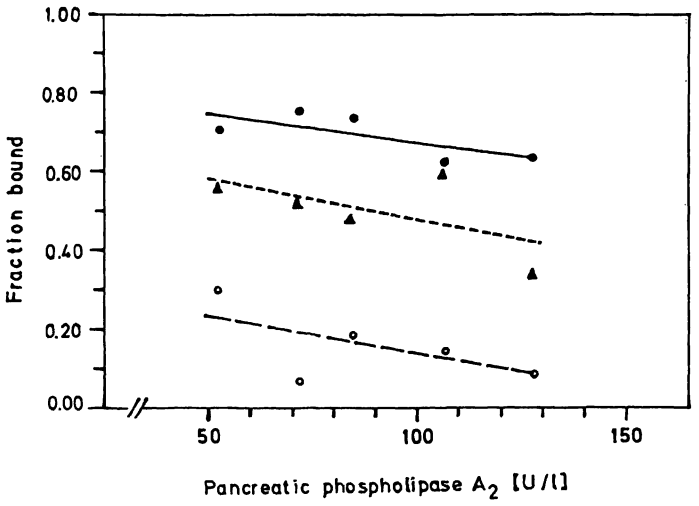

Fig. 1. Binding capacity of two monoclonal antibodies, 2.50.75-IgG-(TSK)-Bi(X-Osu) (closed triangles) and 2.223.288-IgG(Q)-Bi(X-Osu) (closed circles) for purified human pancreatic phospholipase A_2. The closed symbols represent the fraction of specific binding to the monoclonal antibodies, the open circles indicate the fraction of unspecific binding in the absence of antibody. For experimental details see methods section.

the specific binding capacity was 0.6 to 0.8 and the unspecific binding 0.05 to 0.3 (fig. 1). Complete binding was achieved only at barely detectable sample activities around 20 U/l (results not shown). As a compromise, we usually used samples with activities of 50 to 70 U/l, in order to obtain reasonable binding rates under optimal measuring conditions. Calculating a specific activity of 2000 U/mg protein for pancreatic phospholipase A_2 (16), our results indicate that each well was capable of binding 1 to 2 ng antigen in a specific, and 0.5 to 1 ng in an unspecific manner.

In a second series of experiments we tested eight different biological materials as antigens, using five different monoclonal antibodies under the conditions described in the methods section. It is shown (tab. 1) that the proportion of specific binding ranged from 0.7 to 0.9 with purified pancreatic phospholipase A_2, native duodenal juice or the ascites fluid of a patient with necrotizing pancreatitis. Lower and less reproducible binding rates around 0.5 were obtained with a pleural puncture fluid and a serum pool of a patient with necrotizing pancreatitis. Practically no cross-reactivity (0 to 0.13) was seen with partially purified serum phospholipase A_2 and the recombinant platelet enzyme, or with the serum pools of two patients with non-pancreatic diseases (burn injury, septicaemia).

Although the five monoclonal antibodies behaved in a rather similar manner, two of them (designated C and E in the methods section) seemed to be best suited for the differentiation between pancreatic and non-pancreatic phospholipase A_2 in biological materials (see tab. 1).

It is of interest that the tendency of the different phospholipases to bind to the vessel wall in an unspecific manner was very variable, ranging from 0.03 to 0.35 (tab. 1). The lowest values were obtained with purified and native pancreatic phospholipases A_2 and the highest rates with recombinant platelet phospho-

Tab. 1. Specific and unspecific binding of human phospholipase A_2 by 5 different monoclonal antibodies in various biological samples.

Sample	Initial activity U/l	Unspecific binding	Specific binding of monoclonal antibody				
			A	B	C	D	E
Purified pancreatic phospholipase A_2	48	0.04	0.89	0.86	0.91	0.92	0.97
Partially purified serum phospholipase A_2	53	0.11	0	0.01	0	0.04	0
Recombinant platelet phospholipase A_2	58	0.28	0.03	0	0	0.06	0.03
Native duodenal juice	65	0.20	0.82	0.82	0.71	0.77	0.88
Native abdominal exudation (Pat. D)	61	0.03	0.77	0.83	0.88	0.83	0.85
Native pleural exudation (Pat. D)	62	0.13	0.38	0.59	0.66	0.48	0.71
Native serum pool/ necrotizing pancreatitis	48	0.28	0.04	0.17	0.59	0.24	0.18
Native serum pool/severe burns	65	0.35	0.02	0.13	0.05	0	0
Native serum pool/septicaemia	56	0.16	0.01	0	0.07	0.05	0.07

lipase A_2 and two serum pools. The high affinity for surfaces seemed to be more characteristic for the non-pancreatic than the pancreatic isoenzyme. Precoating of microtitre plates with albumin or gelatine instead of poly-*L*-lysine did not reduce this unspecificity (results not shown).

Discussion

The present paper offers a photometric procedure for characterizing phospholipase A_2 isoenzymes in human body fluids (2, 17). Two different human isoenzymes classified as type I and type II phospholipase A_2 have been extensively studied: the pancreatic isoenzyme (type I), which is secreted as an inactive proenzyme and activated by limited proteolysis (18), and an "inflammatory" isoenzyme (type II), which is present as an active protein in human spleen (19), human platelets and arthritic synovial fluid (15). The latter phospholipase is also secreted from cultured liver cells under the control of interleukines and tumour necrosis factor and might act as an acute phase protein, which has been pointed out by *Crowl* et al. (5) and *Matsuda* et al. (19).

Although phospholipases A_2 are well known to be involved in inflammatory processes (4, 7), the discussion about the contribution of different serum isoenzymes to pathological states is still controversial. In acute pancreatitis, it is not clear yet wether the activation of the pancreatic isoenzyme by tryptic autodigestion (18) plays a significant role in the induction of intra- and extra-pancreatic inflammation and necrosis (1, 18). Many phospholipases A_2, e. g. those from snake and insect venoms, are known to be highly toxic (20). The appearance of active phospholipase(s) in blood has been found to be correlated with lethality (4). The evaluation of their possible pathogenetic role is of great scientific and medical interest.

Antibody-based techniques have been used by several investigators in order to prove the presence of pancreatic phospholipase A_2 in human blood and other body fluids (10, 21–23). Since these radio- and fluoroimmunoassays measured the protein mass rather than the activity, it could not be decided whether they detected the active enzyme or inactive precursors and degradation products. *Eskola* et al. (6) used antibody-coated tubes in order to measure specifically the activity of the pancreatic isoenzyme by an immunoabsorption assay. They demonstrated the coexistence of both pancreatic and non-pancreatic phospholipase A_2 activity in serum of patients with pancreatitis.

Using the modified test system described in this article, we have demonstrated the expected presence of pancreatic phospholipase A_2 in duodenal juice and in the ascites of a patient with necrotizing pancreatitis. Furthermore, we were able to show that the pancreatic isoenzyme was absent from the pooled sera of two patients with non-pancreatic inflammatory diseases. It is of interest that in the latter cases unspecific binding of the enzyme was much higher than specific binding. This phenomenon would not be recognized with the original method of *Eskola* et al. (6).

Surprising results were obtained with a pleural exudate and a serum pool of two different patients with necrotizing pancreatitis: in both cases, specific and unspecific binding were incomplete and showed great variability. This observation might be explained by the presence of a mixture of pancreatic and non-pancreatic phospholipase A_2 as already suggested by *Eskola* et al. (6). The assay method described in this article might help to confirm this hypothesis on a larger collective.

In conclusion, the present method seems to be suitable for experimental studies on the diagnostic and pathophysiological role of phospholipase A_2 isoenzymes in patients with pancreatic and non-pancreatic diseases. Our measurements support our previous assumption (1–4) that serum phospholipase A_2 is not exclusively derived from the pancreas. It will be of interest to use the assay described in this article for further investigation of these problems, as well as for a critical re-evaluation of previous studies on the diagnostic and prognostic value of phospholipase A_2 in pancreatitis which have been performed during the last three decades (24–27).

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